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ScienceDirect
Plant Science 173 (2007) 213–222
PLANT SCIENCE
www.elsevier.com/locate/plantsci

# Identification of genes associated with cold acclimation in blueberry (*Vaccinium corymbosum* L.) using a subtractive hybridization approach

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Received 2 April 2007; received in revised form 9 May 2007; accepted 9 May 2007
Available online 16 May 2007

**Abstract**

Enhanced cold tolerance, including tolerance to winter freezing and spring frosts, is needed for genetic improvement of current highbush blueberry (*Vaccinium corymbosum* L.) cultivars. To gain a better understanding of changes in gene expression associated with development of cold tolerance in blueberry and other woody perennials, forward and reverse subtracted cDNA libraries were prepared in such a way to enrich for transcripts that are expressed at higher levels in dormant blueberry flower buds at 400 h and at 0 h of low temperature exposure, respectively. Of the clones picked and single-pass sequenced, 503 clones from the forward subtracted library and 167 clones from the reverse subtracted library had inserts and yielded high quality sequences; and of these, 291 (57.9%) and 51 (31.0%), respectively, were assigned putative identities from BLAST searches of GenBank. From contig analyses to cluster genes of like or identical sequences, 275 unigenes (unique clones) from the forward subtracted library and 99 unigenes from the reverse subtracted library were obtained. Many interesting cold and light-stress related genes were identified from the forward subtracted library and several drought-stress related genes were identified from the reverse subtracted library. Many genes, not previously characterized from blueberry or other woody perennials, encoding putative transcription factors and proteins related to signal transduction were identified from both the forward and reverse subtracted libraries. Eleven genes of interest (six from the forward subtracted library and five from the reverse subtracted library) were selected and their expression was analyzed in floral buds by quantitative real-time RT-PCR over a time course from ~0 to 1200 h of low temperature exposure. Expression profiles validated the quality of the libraries.
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*Keywords:* Cold hardiness; Cold tolerance; Expressed sequence tags; Freezing tolerance; Genomics; Subtracted library

## 1. Introduction

Low temperature combined with insufficient cold hardiness are among the major factors limiting growth and productivity of crop plants. The United States is the world's largest producer of blueberries, an important small fruit crop rich in antioxidants; but enhanced cold hardiness, including tolerance to winter freezing and spring frosts, is needed for genetic improvement of current cultivars [1]. Genetic evidence from numerous plants, including woody perennials such as blueberry, indicates that cold hardiness is a quantitative trait [2–7]. Molecular studies indicate that cold acclimation, or the development of cold hardiness, involves major changes in gene expression. These changes result in the alteration in metabolism and composition of lipids, proteins, and carbohydrates [8–10]. Genes induced during cold stress include those that encode: enzymes required for the biosynthesis of osmoprotectants; lipid desaturases for maintaining membrane fluidity; protective proteins such as antifreeze proteins, dehydrins, chaperones, and mRNA-binding proteins; proteins involved in protein turnover including ubiquitin, ubiquitin-associated proteins, and other proteases; detoxification proteins; and proteins involved in signal transduction such as transcription factors, protein kinases, and phospholipase C [9–12].

There is a need to identify genes associated with cold hardiness in blueberry and use this information to develop more cold hardy cultivars. Toward this direction, our laboratory previously has been using an expressed sequence tag (EST) approach together with microarrays to identify cold acclimation-associated genes from standard cDNA libraries prepared from

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0168-9452/$ – see front matter. Published by Elsevier Ireland Ltd.
doi:10.1016/j.plantsci.2007.05.003

flower bud RNA from cold acclimated and non-acclimated blueberry plants [13,14]. Using this approach, approximately 5.2% of cDNAs imprinted on microarrays have been shown to be upregulated during cold acclimation in the field, and even more (9.4%) shown to be upregulated during acclimation in the cold room environment [14]. Another 6.3% of cDNAs have been shown to be downregulated during cold acclimation in the field [14]. However, random picking and sequencing of even several thousand clones from standard cDNA libraries will result in selection of clones representing more highly abundant transcripts because these clones will be present in the libraries at a higher frequency than those representing less abundant transcripts. Important regulatory genes, such as transcription factors, are often expressed at rather low levels and over a shorter timeframe. Thus, they can be missed using this approach. Many transcription factors have been identified in plants that play key regulatory roles in stress responses, such as AP2/EREBP, MYB/MYC, bZIP, WRKY, and zinc-finger proteins [15]. Therefore, here we report the preparation of forward subtracted and reverse subtracted libraries using procedures that help to increase the chances of finding rarer classes of transcripts by helping to normalize the distribution of clones.

In this work, a forward subtracted library was prepared in such a way to enrich for transcripts that are expressed at higher levels in dormant blueberry flower buds at 400 h of cold acclimation than at 0 h of cold acclimation and vice versa for a reverse subtracted library. Approximately 600 clones from the forward subtracted library and ~300 clones from the reverse subtracted library were picked and single-pass sequenced. Contig analyses and BLAST (NCBI) searches were performed to categorize the genes. From the contig analyses, 275 unigenes (unique clones) from the forward subtracted library and 99 unigenes from the reverse subtracted library were obtained. Of the clones that yielded high quality sequences and had inserts, major percentages (42.1% from the forward subtracted library and 69.5% from the reverse subtracted library) were unidentifiable from BLAST searches, comprising novel and unclassified genes. Using quantitative RT-PCR, expression profiles of mRNAs corresponding to 11 clones, 6 from the forward subtracted library and 5 from the reverse subtracted library, were examined, confirming their differential expression.

## 2. Materials and methods

### 2.1. Construction of forward and reverse subtracted libraries and isolation of plasmid DNA

RNA for library construction was isolated from floral buds of field-grown plants at the Agricultural Research Center (Beltsville, MD, USA) of the highbush blueberry cultivar Bluecrop (*Vaccinium corymbosum* L.) that had accumulated 0 and 400 chill units. Bluecrop was chosen because it is quite cold hardy and is the ''industry standard'' of highbush cultivars. One chill unit, as defined here, equals 1 h of exposure to temperatures between 0 and 7 °C. The 0 and 400 chill unit time points corresponded to collection times on 29 September 2003 and 8 December 2003, respectively, and cold hardiness levels, expressed as lethal temperature$_{50}$ ($LT_{50}$) or temperature that kills 50% of the floral buds in a controlled freeze-thaw test, of −10 and −25 °C, respectively [16]. Late September is about the earliest time flower buds can be collected from Bluecrop, when plants have set sufficient numbers of buds and flower buds can be distinguished from leaf buds. For Bluecrop, maximum cold hardiness level of −27 °C is generally reached midwinter (January to February), after accumulating about 800 chill units [14,16]. Total RNA was extracted from ~600 mg frozen floral bud samples using the 'hot borate' protocol outlined by Wilkins and Smart [17]. Total RNA (5 μg/lane) from each time point was separated on 1% agarose/formaldehyde gels, visualized and photographed to confirm concentration and quality.

Total RNA was provided to Virotech International, Inc. (Gaithersburg, MD, USA) for construction of the forward and reverse subtracted libraries. First, to remove any genomic DNA contamination, total RNA was treated with RNAse-free DNAse I (Promega Corporation, Madison, WI, USA). Briefly, the total RNA was mixed with DNAse I (1 unit/μg of total RNA) in 1× reaction buffer and incubated at 37 °C for 1 h. After incubation, the DNAse I activity was terminated by adding EDTA and five volumes of guanidine thiocyanate (6 M) solution. The RNA solutions were re-purified over glass bead-based micro-spin columns provided in Ambion's plant RNA aqueous kit (Ambion Inc., Austin, TX, USA). Columns were washed with ethanol-based washing solution, also provided in the kit, two times followed by elution in DEPC-treated nuclease-free water. RNA was quantified spectrophotometrically by taking the absorbance at 260 nm and quality was again assessed on 1% agarose/formaldehyde gels.

The two subtracted cDNA libraries, forward and reverse, were constructed through reciprocal suppressive subtractive hybridization using the PCR-Select cDNA subtractive hybridization kit essentially according to the manufacturer's protocol (Clontech, USA). For the forward subtracted library, cDNA from the 400 chill unit flower buds was used as tester and cDNA from the 0 chill unit flower buds was used as driver, and vice versa for the reverse subtracted library. Several rounds of suppressive subtractive hybridization were carried out to reduce the number of common transcripts. The subtracted cDNA products were ligated into pCR4 TOPO® sequencing vector (Invitrogen Life Technologies, Carlsbad, CA, USA) and recombinants selected by alpha complementation.

About 600 clones from the forward subtracted library and 300 clones from the reverse subtracted library were randomly selected for sequencing. Clones were grown and plasmid DNAs were isolated using Edge Biosystems' 96-well plasmid DNA isolation kit (Gaithersburg, MD, USA). Plasmid DNA was quantified spectrophotometrically.

### 2.2. DNA sequencing and analysis of sequence data

Single-pass nucleotide sequencing of recombinant plasmid DNAs was performed by Virotech International, Inc. using the M13R primer. Resulting DNA sequences were trimmed of vector sequence using the software package 'Lasergene' (DNASTAR Inc., Madison, WI, USA). Sequences were compared with the

National Center for Biotechnology Information (NCBI) non-redundant protein database (http://www.ncbi.nlm.nih.gov/BLAST/) using BLASTX [18] and default parameters. Sequences with no significant similarity with sequences in the protein database were compared with the nucleotide database using BLASTN. Individual ESTs from each library were assembled into contigs using 'Lasergene'. Consensus sequences from the contig analyses were also compared with the non-redundant protein database using BLASTX. The highest BLAST scores from either the individual ESTs or contigs were used to assign putative identities to the clones. In general, the sequences with BLASTX similarity scores >100 and probabilities $<10^{-5}$ were considered significant [19–21].

ESTs were classified into 14 functional groups and subgroups as described previously for grape [22] and blueberry [13]. Classification was based on the authors' knowledge of biochemistry, plant physiology, and plant molecular biology, by reference to the BioCyc-MetaCyc: Encyclopedia of Metabolic Pathways website (http://MetaCyc.org/) or the gene ontology (Go) database (http://www.geneontology.org/), and by searching related abstracts in PubMed (http://www.ncbi.nlm.nih.gov/PubMed/).

### 2.3. Quantitative real-time RT-PCR

RNA for RT-PCR was extracted from floral buds of Bluecrop field plants that had accumulated 0 (collected 29 September 2003), 67 (20 October 2003), 399 (8 December 2003), 779 (2 February 2004), and 1234 (18 March 2004) chill units. RNA was extracted from ~600 mg frozen samples using the 'hot borate' protocol described by Wilkins and Smart [17]. To remove any genomic DNA contamination, total RNA was treated with RNAse-free DNAse I (Promega Corporation) as described above. Total RNA (5 μg/lane) from each time point was separated on 1% agarose/formaldehyde gels, visualized and photographed to confirm quality and concentration.

cDNA was synthesized from RNA using the Superscript™ III Platinum® Two-Step qRT-PCR SYBR kit (Invitrogen) according to the manufacturer's instructions. Primers were designed from ESTs of the genes of interest and housekeeping genes using the P3 website (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi), and to have a melting temperature of 59 ± 1 °C and amplify products of ~250 bp. cDNA and primers were first tested in standard PCRs followed by gel electrophoresis to verify that they amplified a single product of the correct size. If they did not, the primers were redesigned and tested again.

Quantification of mRNA expression of 11 selected genes (6 from the forward and 5 from the reverse subtracted library) at different chill units was performed by quantitative real-time RT-PCR using the SYBR Green RT-PCR kit and were run on the i-Cycler by Bio-Rad (Hercules, CA, USA). Quantification and expression analysis was performed with Gene Expression Analysis for iCycler iQ® Real-Time PCR Detection System software. The program used was as follows: 95 °C for 3 min followed by 50 cycles of 95 °C 10 s denaturing and 58 °C for 60 s annealing/extension. Amplicon dissociation curves (melting curves) were determined after cycle 50 by heating from 58 to 95 °C with a ramp speed of 0.5 °C/min. Dissociation curves for each amplicon were carefully examined for presence of a single dissociation peak, and only data from those runs were selected for analysis. Efficiency of real-time RT-PCR was determined by comparison of experimentally determined and theoretically expected threshold values in dilution series of cDNA using 125, 62.5, 31.25, and 15.625 ng per reaction. Correlation coefficients of >0.999 were routinely obtained and used for normalization of experimental data.

Relative expression ratios were calculated as the ratios of normalized gene expression of the genes of interest (GOI) against a strongly, constitutively expressed housekeeping gene (HKG). Metallothionein (clone NA186 from our standard non-acclimated cDNA library) was selected as HKG based on previous northern [13] and microarray data [14]. Other constitutively expressed genes (clones CA1110, CA1381, CA1389, and NA1791) that were identified from microarray data [14] were used in some runs with GOI to confirm their expression levels (data not shown). The following formula was used for expression studies: relative expression ratio of the GOI is $2^{-\Delta Ct}$ where $\Delta Ct = Ct_{GOI} - Ct_{HKG}$ and Ct denotes the threshold cycle [23]. All experiments were repeated at least two times on cDNA prepared from different batches of RNA. Deviations of threshold values were less than two cycles for most of the replicated mean Ct values.

## 3. Results and discussion

### 3.1. Quality of the libraries and sequences

The quality of the forward and reverse subtracted libraries and DNA sequences, as assessed by insert size, percent of sequences with <2% Ns, average length of sequences, etc. is summarized in Table 1. To verify the presence and determine the range of sizes of inserts, plasmid DNA from about 20 clones from each of the forward and reverse subtracted libraries was digested with EcoRI and fractionated on agarose gels. Both libraries appeared to be of reasonable quality having inserts that ranged in size from 0.5 to >1.5 kb. Single-pass sequencing of the clones resulted in high quality sequences (<2% Ns) from 96.8 and 80.3% of the clones from the forward subtracted and reverse subtracted libraries, respectively. After trimming vector sequences, the average read-length was 608 bases from the forward subtracted library and 646 bases from the reverse subtracted library. From the high quality sequences, approximately 11.8 and 27.9% of the clones from the forward and reverse subtracted libraries, respectively, appeared to lack inserts or to have inserts that were too small to obtain meaningful BLAST results. The ESTs generated from clones with inserts were deposited into GenBank.

### 3.2. Contig assembly

The software package 'Lasergene' was used to assemble the ESTs from each library into contigs or clusters based on the presence of overlapping, identical, or similar sequences. The

Table 1
Quality of forward and reverse subtracted libraries and DNA sequences

| | Forward subtracted | Reverse subtracted |
|---|---|---|
| Number of clones picked | 589 | 290 |
| Range of insert sizes (kb) | 0.5 → 1.5 | 0.5 → 1.5 |
| Number of clones yielding high quality sequences | 570 | 233 |
| Percent high quality sequences | 96.8% | 80.3% |
| Average sequence length (bases) | 608 | 646 |
| Fraction of vector-only sequences (percent of vector-only sequences out of total high quality sequences) | 67/570 (11.8%) | 66/233 (28.3%) |

program was run with default threshold settings for clustering sequences with at least 80% similarity or with 12 or more overlapping consecutive bases. The ESTs from the forward subtracted library yielded 213 singletons and 62 contigs comprised of from 2 to 42 sequences. The ESTs from the reverse subtracted library yielded 43 singletons and 56 contigs comprised of 2–7 sequences. The average length of the contigs from the forward subtracted library was 693.5 and 678.5 bp from the reverse subtracted library.

Contig analysis was also carried out after combining all the ESTs from both libraries. In this case, 254 singletons and 118 contigs were formed, representing a total of 372 distinct transcripts. Of the 118 contigs, 50 included sequences from the forward subtracted library only, 57 included sequences from the reverse subtracted library only, and only 11 contigs included sequences from both libraries. As expected, a very low percentage, ~3% (11/372), of the total distinct transcripts were shared between the libraries.

### 3.3. Overview of BLAST results

Over half of the ESTs from the forward subtracted library and about a third of the ESTs from the reverse subtracted library could be assigned putative functions on the basis of sequence similarity to genes or proteins of known function in GenBank. Of the 503 clones from the forward subtracted library and the 167 clones from the reverse subtracted library that yielded high quality sequences and had inserts, 291 (57.9%) and 51 (31.0%), respectively, were assigned putative identities. The sequences from the remaining clones showed either significant similarity to protein or DNA sequences that were of unknown function (34/503 or 6.8% for the forward subtracted library and 32/167 or 19.0% for the reverse subtracted library) or no significant similarity to any other sequences in the databases (178/503 or 35.4% for the forward subtracted library and 84/167 or 50.0% for the reverse subtracted library).

### 3.4. Highly abundant cDNAs/transcripts from each library and their functional classification

Of those clones that could be identified from GenBank searches, the most highly abundant ones (clones that were picked at random three or more times) from the forward subtracted and reverse subtracted libraries and their functional classifications are presented in Tables 2 and 3, respectively. For comparison, the number of times the same type of clone was picked and the percentage of each type of clone from the other library are shown in parentheses. Picking a clone many more

Table 2
Most abundant clones from the forward subtracted library

| Putative gene identification | # of cDNAs[a] | Percent of cDNAs[b] | Functional category |
|---|---|---|---|
| Early light-inducible protein | 155 (0)[c] | 30.8 (0) | Cell structure/chloroplast |
| Beta-amylase | 14 (0) | 2.8 (0) | Primary metabolism/sugars and polysaccharides |
| Dehydrins | 13 (0) | 2.6 (0) | Stress responses |
| Ribulose 1,5 bisphosphate carboxylase/oxygenase, small subunit | 11 (3) | 2.2 (1.8) | Photosynthesis |
| Late embryogenesis abundant (LEA) proteins | 9 (0) | 1.8 (0) | Stress responses |
| Galactinol synthase | 8 (0) | 1.6 (0) | Primary metabolism/sugars and polysaccharides |
| Proline-rich proteins | 8 (0) | 1.6 (0) | Cell structure/cell wall |
| NADH dehydrogenase chain | 6 (0) | 1.2 (0) | Energy |
| Zinc-finger proteins | 6 (1) | 0.8 (0.6) | Transcription factors |
| Extensin | 5 (0) | 1.0 (0) | Cell structure/cell wall |
| Nodulin-24 | 5 (0) | 1.0 (0) | Development |
| Seed maturation family proteins | 5 (0) | 0.8 (0) | Stress responses |
| Ion transporters | 4 (0) | 0.8 (0) | Transporters |
| bZIP family transcription factors | 3 (0) | 0.6 (0) | Transcription factors |
| F1 ATPase subunit alpha | 3 (0) | 0.6 (0) | Energy |

[a] Number of times a particular cDNA was picked from the forward subtracted library.
[b] Percent of clones out of 503, the total number of clones with inserts yielding high quality sequences from the forward subtracted library.
[c] In parentheses is the number of times a particular cDNA was picked and the percentage of a particular cDNA from the reverse subtracted library for comparison.

Table 3
Most abundant clones from the reverse subtracted library

| Putative gene identification | # of cDNAs[a] | Percent of cDNAs[b] | Functional category |
|---|---|---|---|
| Anthocyanidin reductases | 5 (0)[c] | 3.0 (0) | Secondary metabolism/phenyl-propanoids |
| Protein kinases | 4 (1) | 2.4 (0.2) | Kinases |
| CBF (C-repeat binding factor) | 4 (0) | 2.4 (0) | Transcription factors |
| Other AP2-domain proteins | 2 (0) | 1.2 (0) | Transcription factors |
| Mitochondrial uncoupling proteins | 3 (0) | 1.8 (0) | Energy |
| GDSL-motif lipase/hydrolase | 3 (1) | 1.8 (0.2) | Primary metabolism/lipids |
| Ribulose 1,5 bisphosphate | 3 (11) | 1.8 (2.2) | Photosynthesis carboxylase/oxygenase, small subunit |
| Chitinase | 3 (1) | 1.8 (0.2) | Defense-related |

[a] Number of times a particular cDNA was picked from the reverse subtracted library.
[b] Percent of clones out of 167, the total number of clones with inserts yielding high quality sequences from the reverse subtracted library.
[c] In parentheses is the number of times a particular cDNA was picked and the percentage of a particular cDNA from the forward subtracted library for comparison.

times from one library than from the other provides supportive evidence for the clone representing a differentially expressed transcript.

The clones that were picked by far the most number of times (155 times) from the forward subtracted library encoded early light-inducible protein or ELIP, a protein believed to be involved in protection of photosystem II against light stress [24,25] (Table 2). These clones represented 30.8% of the total number of usable clones picked (having inserts yielding high quality sequences) from this library. We have previously shown from our standard EST library and microarray work that ELIP-message levels are maximally upregulated almost nine-fold during cold acclimation of blueberry field plants [13,14]. Under field conditions in winter, blueberry floral buds are likely exposed to excessive light. In our experiments, the buds we collect are the whole buds, including bud scales. The light energy harvested by floral buds may exceed what can be processed by photosystems in winter, when expression of photosynthesis-related genes are likely turned down, making them vulnerable to photoinhibition and photooxidative damage. Various protective mechanisms allow plants to survive light stress, including a rise in ELIP level, which is a nuclear-encoded thylakoid membrane protein that was originally found to be transiently induced during greening of etiolated plants [26]. ELIPs bind chlorophyll *a* and lutein, and are speculated to protect chloroplasts from light-induced damage by functioning as photoprotective pigment carriers or chlorophyll exchange proteins [27]. In addition, they might provide protection against photooxidative damage through the dissipation of excessive light energy [28].

Other highly abundant clones from the forward subtracted library that encode cold stress-response proteins included dehydrin, LEA, and seed maturation family cDNAs, none of which were picked at all from the reverse subtracted library. Highly abundant clones encoding proteins involved in sugar and starch metabolism included cDNAs for beta amylase and galactinol synthase; these, too, were not picked from the reverse subtracted library. All of these genes have been shown to be cold induced in *Arabidopsis* [11,12,29] and were identified as being cold induced in blueberry under both field and cold room conditions in our recent microarray experiments [14]. Highly abundant clones encoding cell wall proteins included cDNAs for a proline-rich protein and extensin. Messages of this type were not identified as cold induced in blueberry from our previous microarray experiments [14] but have been identified by other researchers as cold induced in *Arabidopsis* [29]. Other interesting highly abundant clones from the forward subtracted library included cDNAs encoding ion transporters and zinc-finger and bZIP family transcription factors. Some messages of each of these three families have been shown to be cold induced in *Arabidopsis* [29] and in blueberry [14] from previous microarray experiments. These results support the conclusion that the forward subtracted library is indeed enriched for genes whose expression is upregulated during cold acclimation, when plants are nearing maximal cold tolerance.

In the reverse subtracted library, no single clone represented 30%, or even more than 10%, of the usable cDNAs that were picked, like the ELIP-cDNAs did from the forward subtracted library. The most highly abundant type of clone from the reverse subtracted library comprised 3.0% of the usable clones picked and encoded anthocyanidin reductases (Table 3). This is consistent with the observation that blueberry plants accumulate anthocyanins in the fall in response to cooler temperatures, and turn bright red [30].

The next most highly abundant clones from the reverse subtracted library, comprising 2.4% each, encoded a variety of different protein kinases and the CBF or C-repeat binding factors. The CBF proteins are transcription factors, originally discovered in *Arabidopsis*, that are induced within 15 min of cold stress and responsible for the upregulation of many genes in response to cold and drought stress [12,31–34]. Our previous efforts to isolate a CBF clone from blueberry, using (1) specific and degenerate primers designed from the most highly conserved *CBF* sequences from other plants and (2) the *CBF1* gene from *Arabidopsis* as a probe to screen our standard cDNA library from flower buds of cold acclimated plants and on Southern blots had all failed (data not shown). Finding CBF clones to be one of the more highly abundant clones in our reverse subtracted library was surprising. However, cold acclimation in woody perennials is more complex than that in herbaceous annuals. In woody perennials, cold acclimation is considered basically a two-step process [35]. The first stage is induced by short photoperiod and the timing and speed of acclimation can be affected by other factors such as available moisture. The second stage is induced by low temperature. Finding CBF-messages in our 0 chill unit control plants

(exposed for 0 min between 0 and 7 °C) indicates that conditions under which temperatures still remained above 7 °C induced CBF expression in blueberry. CBF may have been induced by gradually declining temperatures and/or shortening photoperiods, by some critical temperature above 7 °C, or by some other factor. To the best of our knowledge, however, CBF has not been shown to be induced by short photoperiods in other plants. A more likely explanation may be that CBF is induced by dehydrating conditions in floral bud tissue in the fall. Indeed, several other clones from the reverse subtracted library encoded proteins potentially related to drought stress and/or abscisic acid signaling (discussed in more detail under Section 3.5). Wake and Fennell [36] reported that water content decreases in grape buds with short-day treatments and may be a necessary factor in dormancy induction. CBF is also known as DREB (dehydration responsive element-binding protein) as it is induced by drought [33] as well as cold. Isolating these partial-length CBF clones have enabled us to clone a full-length *CBF1* gene from blueberry which will be described in detail elsewhere (manuscript in preparation). It is worth noting that CBFs are AP2 domain-containing proteins [31–33] and two clones encoding other AP2 domain-containing proteins were isolated as well from the reverse subtracted library.

Other highly abundant clones that were picked more times from the reverse subtracted library than from the forward subtracted library included cDNAs for mitochondrial uncoupling proteins, GDSL-motif lipase/hydrolase, and chitinase. Chitinases have been shown to be responsive to a number of different stresses, both biotic and abiotic, and to different phytohormones [37]. Overexpression of a gene encoding a GDSL-motif family lipase, *AtLTL1*, has recently been shown to increase salt tolerance in transgenic *Arabidopsis* plants [38]. Mitochondrial uncoupling proteins in plants termed PUMPs are thought to play a role in protecting plant cells from oxidative stress. Some *Arabidopsis* PUMP genes (*AtPUMP4* and *AtPUMP5*) are induced rapidly and transiently upon exposure to low temperature whereas others are not responsive to cold stress [39].

### 3.5. Less abundant cDNAs/transcripts from each library

Of those clones that could be identified from GenBank searches, a selected subset from each of the forward subtracted and reverse subtracted libraries are listed in Table 4 along with their GenBank accession numbers. Those shown were of the most interest to us either because of their putative role in protection against cold/drought stress or abiotic stress in general or because they may encode regulatory genes such as signaling molecules and transcription factors. Highly abundant and less abundant ones are listed. For the highly abundant ones, only one or a few representative clone names and accession numbers are given. A list of all ESTs generated from each of the libraries and their BLAST results are provided on our website, http://psi081.ba.ars.usda.gov/BBGD/index.htm, which houses the blueberry genomics database.

Of particular interest from the forward subtracted library, besides the previously mentioned highly abundant clones, were

Table 4
A selected subset of clones from each of the forward and reverse subtracted libraries and their GenBank accession numbers

| Putative gene identification | Clone name | Accession number |
|---|---|---|
| Forward subtracted library | | |
| ABA-induced membrane protein | SL282 | DW043270 |
| Beta-amylase | SL551 | DW043515 |
| $Ca^{2+}/H^{+}$ exchanging protein | SL9 | DW043562 |
| $Ca^{2+}$-transporting ATPase | SL385 | DW043381 |
| Calmodulin-binding family protein | SL236 | DW043225 |
| Calmodulin-related protein | SL69 | DW043542 |
| Chlorophyll *a*/*b*-binding protein type III | SL269 | DW043255 |
| Class III HD-Zip protein 2 | SL426 | DW043423 |
| Dehydrin | SL506 | DW043484 |
| DNA-binding protein | SL384 | DW043380 |
| DnaK-type molecular chaperone LIM18 | SL116 | DW043102 |
| Dormancy-associated protein | SL290 | DW043279 |
| Early light-inducible protein | SL214 | DW043206 |
| Extensin | SL297 | DW043286 |
| Fatty acid desaturase (COG1398) | SL483 | DW043467 |
| Galactinol synthase | SL343 | DW043337 |
| Gibberellin-regulated protein | SL386 | DW043382 |
| Glutathione-*S*-transferase | SL395 | DW043391 |
| Late embryogenesis abundant (LEA) proteins | SL326 | DW043319 |
| Leaf senescence-related protein | SL18 | DW043170 |
| Lipocalin protein | SL374 | DW043370 |
| Low temperature and salt-responsive protein | SL452 | DW043444 |
| Nodulin-24 | SL272 | DW043259 |
| Omega-3-fatty acid desaturase | SL45 | DW043441 |
| Photosystem I P700 apoprotein A2 | SL565 | DW043525 |
| Photosystem II 10 kDa phosphoprotein | SL276 | DW043263 |
| Photosystem II CP47 protein | SL377 | DW043373 |
| Potassium transporter | SL317 | DW043309 |
| Proline-rich proteins | SL383 | DW043515 |
| Protein kinase family protein | SL65 | DW043538 |
| Protein phosphatase PP1 | SL400 | DW043398 |
| Putative disease-resistance protein | SL11 | DW043096 |
| Putative myb-related protein | SL417 | DW043413 |
| Putative ripening-related bZIP protein | SL190 | DW043182 |
| Seed maturation family proteins | SL89 | DW043561 |
| Transducin family protein/WD-40 repeat family | SL267 | DW043253 |
| Zinc-finger proteins | SL4 | DW043396 |
| | SL26 | DW043246 |
| | SL353 | DW043348 |
| Reverse subtracted library | | |
| Annexin | RL73 | DW043071 |
| Anthocyanidin reductases | RL130 | DW043009 |
| Basic helix-loop-helix transcription factor | RL49 | DW043058 |
| BURP domain-containing protein RD22 | RL89 | DW043079 |
| Calmodulin | RL143 | DW043018 |
| CBF proteins | RL44 | DW043054 |
| | RL82 | DW043077 |
| Other AP2 domain-containing proteins | RL24 | DW043040 |
| | RL101 | DW042990 |
| Class IV chitinases | RL4 | DW043050 |
| COP8-like protein | RL76 | DW043072 |
| Dihydroflavonol-4-reductase | RL80 | DW043076 |
| Expansin | RL28 | DW043044 |
| GDSL-motif lipase/hydrolase | RL63 | DW043067 |
| Metallothionein-like protein | RL21 | DW043035 |
| Mitochondrial uncoupling protein 4 | RL117 | DW042998 |
| Mitochondrial uncoupling protein 5 | RL60 | DW043066 |

Table 4 (*Continued*)

| Putative gene identification | Clone name | Accession number |
|---|---|---|
| Mitogen-activated protein kinase 1 | RL10 | DW042988 |
| PERK1-like protein kinase | RL9 | DW043080 |
| P-protein-like protein | RL124 | DW043004 |
| Putative calcium-binding protein | RL35 | DW043047 |
| Putative DNA-binding protein | RL22 | DW043037 |
| Putative WD-repeat protein | RL129 | DW043007 |
| RING zinc-finger protein | RL51 | DW043061 |
| Secretory carrier membrane protein | RL109 | DW042994 |
| Transcriptional activator RF2a | RL15 | DW043023 |
| Ubiquitin-conjugating enzyme 2 | RL116 | DW042997 |

many clones encoding cold stress related proteins such as glutathione-*S*-transferase, putative fatty acid desaturase (COG1398), omega-3-fatty acid desaturase, low temperature and salt-responsive protein, leaf senescence-related protein, and dormancy-associated protein. These are proteins that have been shown previously to be associated with cold stress responses either from our own work on blueberry [13,14] or from work on other plants [29]. Also from the forward subtracted library were clones encoding other potentially light-stress related proteins, in addition to the previously mentioned highly abundant early light-inducible protein. These included several photosystem I and II-related proteins, such as photosystem I P700 apoprotein A2, photosystem II 10 kDa phosphoprotein, and photosystem II CP47 protein. From our microarray work, we previously found the message encoding photosystem II CP47 protein to be upregulated in blueberry flower buds during cold acclimation in the field [14]. Many clones encoding putative transcription factors and other proteins related to signal transduction were also present such as the zinc-finger proteins previously mentioned, several calcium-transport related proteins, calmodulin-related protein, calmodulin-binding family protein, ripening-related bZIP protein, putative myb-related protein, class III HD-Zip protein 2, protein kinase family protein, DNA-binding protein, and protein phosphatase PP1. Calmodulin [40], zinc-finger [41,42], bZIP [43,44], and myb-related proteins [45] have all been shown to play roles in cold stress signaling in herbaceous plants. Finding them in blueberry will allow us to test their function in cold acclimation in a woody perennial.

An abundance of clones encoding putative transcription factors and other proteins related to signal transduction were also present in the reverse subtracted library. Besides clones encoding the highly abundant AP2 domain-containing proteins such as CBF, there were clones encoding mitogen-activated protein kinase 1, PERK1-like protein kinase, calmodulin, putative calcium-binding protein, putative WD-repeat protein, COP8-like protein, RING zinc-finger protein, putative DNA-binding protein, basic helix-loop-helix transcription factor, and transcriptional activator RF2a. All of these are potentially quite interesting and warrant further investigation. For example, basic helix-loop-helix transcription factors have been shown to play roles in the activation of structural genes involved in anthocyanin biosynthesis [46]. They have also been shown to play roles in light signal transduction [47] and in abscisic acid-mediated response [48]. In addition, ICE1, inducer of CBF Expression 1, is a MYC-like basic helix-loop-helix protein [49]. Another interesting protein, COP8, is a subunit of the COP9 signalosome [50], which is involved in mediating light control of development. Several other clones from the reverse subtracted library encoded proteins potentially related to drought stress and/or abscisic acid signaling, such as BURP

Table 5
Primer sequences designed from a selected subset of genes from each of the forward and reverse subtracted libraries, as well as housekeeping genes identified from previous northerns and/or microarray data, for use in quantitative real-time RT-PCR

| Putative gene identification | Clone name | Primer sequences[a] |
|---|---|---|
| Forward subtracted library | | |
| $Ca^{2+}/H^{+}$ exchanging protein | SL9 | F-CTGAAGAATCCCCCAGATGA; R-ATCTCCTTGAAACCGGCTCT |
| Dormancy-associated protein | SL290 | F-GAAGGAGAGGGAAGCAAGGT; R-TGGGTGTGGCTTGTCAAATA |
| Putative myb-related protein | SL417 | F-CACTGCCAGTAGTTCGAGCA; R-TACTGTCCTCAGCCATGCAG |
| Zinc-finger proteins (B-box type) | SL4 | F-AGACGTGAGCCTTGTTTGCT; R-TCGTGTTGTGGGAGGTACAA |
| | SL26 | F-CACGAACCACCAACAACAAC; R-CCCTGATCCTTGTTTCAACG |
| | SL353 | F-TTATCCGAAACTCGGTGGAG; R-GCAAACCCCCTTAACGAAAT |
| Reverse subtracted library | | |
| Anthocyanidin reductase | RL130 | F-GTTAGGGATCCCGACAATCA; R-GCCGGCTTTATCATGTCATT |
| AP2 domain-containing proteins | RL24 | F-TTACATCACCGTCGAGTGGA; R-TCCCGAAAACAGAGTCACCT |
| | RL101 | F-CTTTCAACCCATCGCATTCT; R-AGGTAGCTGTTGCGGATTTG |
| Dihydroflavonol-4-reductase | RL80 | F-GGCCGAGGTACTTGAACTGA; R-TTTGAGCATCCTGAAGCAGA |
| Putative calcium-binding protein | RL35 | F-GTACGCGGGGACTGAAATTA; R-AGCTCGGAGACCGAGATTTT |
| Housekeeping genes | | |
| AP2 domain-containing protein | CA1110 | F-GATGTGGGACCTGTTGAAGC; R-TCCAGCAACCAAATCATCAA |
| Asparaginyl tRNA-synthase | CA1381 | F-CAAGTACCCCTTGCCAAAGA; R-CTCCTGCACCTTCACAATCA |
| Katanin | CA1389 | F-AGCAAAGGCTGCTGGTTCTA; R-CCAAGACATCCCTTTCAAGC |
| Metallothionein | NA186 | F-ACCCTGACATGAGCTTCTCG; R-ACCCAAATCTCTGCTTGCTG |
| Pumilio-domain-containing protein | NA1791 | F-CTTTCCTGCCTCACCTCTTG; R-GGCATTGCTGGATTTCAACT |

[a] Forward and reverse primer sequences are given and abbreviated F and R, respectively.

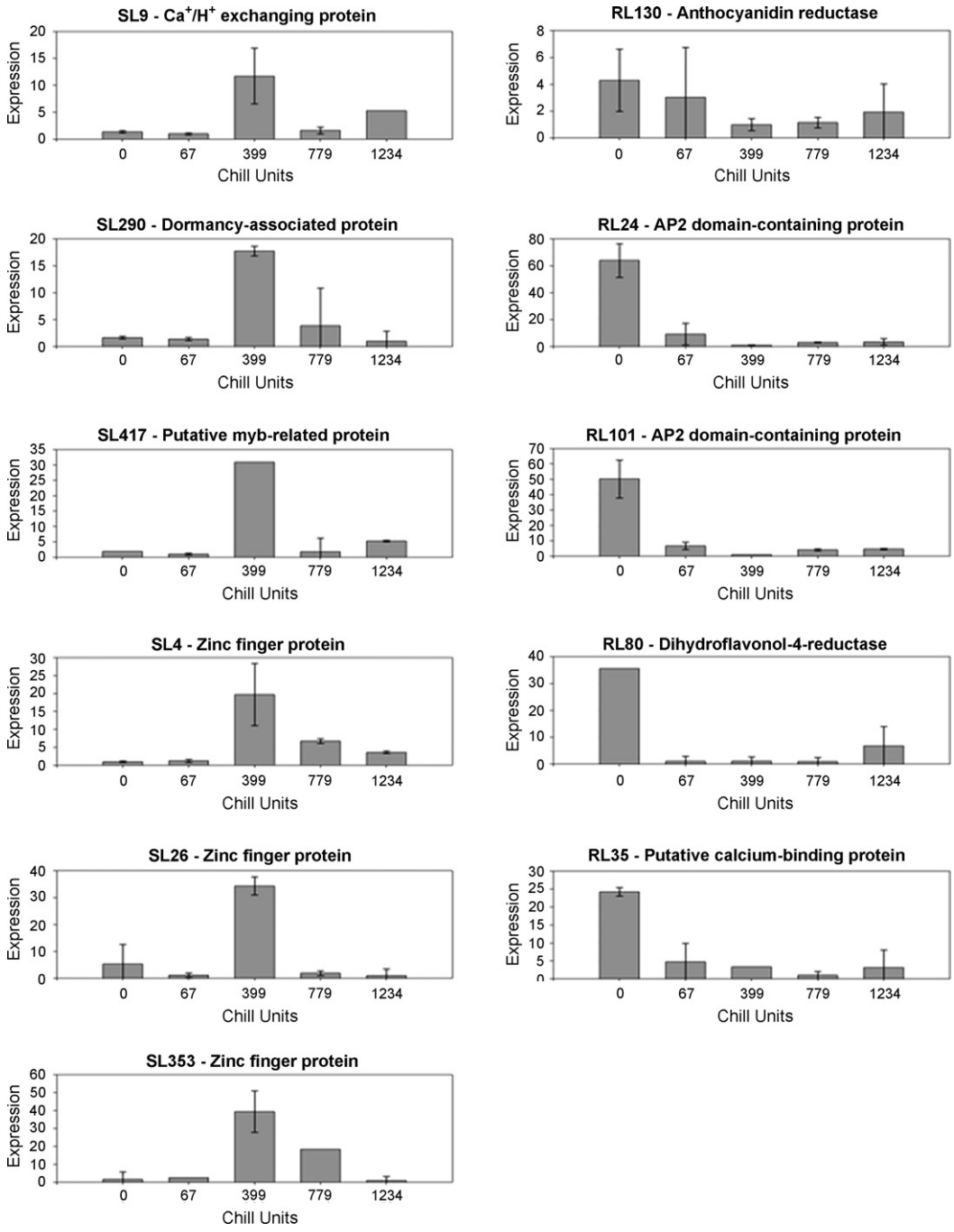


Fig. 1. Quantitative real-time RT-PCR was performed to validate the quality of the forward and reverse subtracted libraries. Eleven genes of interest or GOIs (six from the forward subtracted library and five from the reverse subtracted library) were selected and expression of their transcripts was analyzed in floral buds of Bluecrop over a time course from ∼0 to 1200 chill units. Expression levels, derived from mean threshold cycles or Ct values and expressed relative to the housekeeping gene or HKG metallothionein, are graphed vs. chill units for each of the GOIs.

domain-containing protein RD22 [48], annexin [51], expansin [52], and a RING zinc-finger protein. XERICO is an *Arabidopsis* RING zinc-finger protein recently shown to confer drought tolerance through increased abscisic acid biosynthesis [53].

### *3.6. Validation of forward and reverse subtracted libraries by quantitative real-time RT-PCR*

To validate the quality of the forward and reverse subtracted libraries, 11 genes of interest or GOIs (6 from the forward subtracted library and 5 from the reverse subtracted library) were selected and their expression was analyzed in floral buds over a time course from ∼0 to 1200 chill units by quantitative real-time RT-PCR. For each of the genes, real-time RT-PCR was repeated for each time point at least two times using cDNA prepared from different batches of RNA. The clones from each of the libraries, in addition to clones representing several housekeeping genes or HKGs (based on previous northern blot and/or microarray data [13,14]) are listed in Table 5, along with the primer sequences that were used. Primers were designed to

yield amplification products of ~250 bp and were first tested in standard PCRs to verify that they amplified a single product of the correct size. Metallothionein (clone NA186 from our standard non-acclimated cDNA library) served as the HKG in all runs. Other HKGs were included in some runs to verify the expression patterns of the GOIs.

Expression levels were calculated from mean threshold cycles or Ct values expressed relative to the HKG metallothionein, for each of the GOIs at each of the time points. Expression levels are graphed versus chill units and shown in Fig. 1. All six genes from the forward subtracted library exhibited higher expression levels at 399 chill units than at 0 chill units, while all five genes from the reverse subtracted library showed higher expression levels at 0 chill units than at 399 chill units, as would be expected. In the case of the forward subtracted library, expression of all of the analyzed genes appeared to peak at 399 chill units and to have declined by 779 chill units. From our previous microarray data [14], most of the cold acclimation-induced genes peaked at 399 or 779 chill units and declined thereafter. By 1234 chill units, in fact, floral buds were beginning to deacclimate. In the case of the reverse subtracted library, the analyzed genes appeared to vary in terms of when the minimum expression level was reached, with some reaching a minimum at 67 min, some at 399 min, and some at 779 h. The differential expression levels at 0 and 399 chill units, however, confirmed that both the forward and reverse subtracted libraries were of good quality, enriched for genes that were cold acclimation-induced or cold acclimation-suppressed by 400 chill units, respectively.

Here, we have used a genomic approach based on construction of forward and reverse subtracted libraries to identify cold acclimation-responsive genes in blueberry flower buds. As one would expect, some of the genes identified were the same or similar to genes identified from our standard cDNA libraries and microarray analyses. However, as we had hoped and different from our previous standard libraries, an abundance of clones encoding putative transcription factors and other proteins related to signal transduction were identified in both the subtracted libraries. These include zinc-finger, basic helix-loop-helix, bZIP, myb-related, and AP2 domain-containing proteins, as well as a variety of protein kinases and proteins involved in calcium signaling. These genes will be useful in dissecting cold acclimation pathways in woody perennials, for which research is lacking. For example, finding CBF clones in the reverse subtracted library, before exposure to temperatures below 7 °C, suggests CBF may be induced in woody perennials in the first stage of cold acclimation by dehydrating conditions in dormant flower buds prior to exposure to cold. In addition, many cold and light-stress related genes were identified from the forward subtracted library and several drought-stress related genes were identified from the reverse subtracted library. Furthermore, many of the clones from both the forward and reverse subtracted libraries were unidentifiable from BLAST searches, suggesting that they represent novel, unclassified genes, perhaps unique to blueberry or other woody perennials. Quantitative real-time RT-PCR, performed on selected genes, validated the quality of both the forward and reverse subtracted libraries. Thus, our results indicate that subtractive hybridization is an effective strategy for identifying genes involved in the cold acclimation pathway in woody perennials.

## References


[1] J.N. Moore, The blueberry industry of North America, Acta Hort. 346 (1993) 15–26.

[2] P.M. Hayes, T. Blake, T.H.H. Chen, S. Tragoonrung, F. Chen, A. Pan, B. Liu, Quantitative trait loci on barley (*Hordeum vulgare* L.) chromosome 7 associated with components of winterhardiness, Genome 36 (1993) 66–71.

[3] J.M. Stone, J.P. Palta, J.B. Bamberg, L.S. Weiss, J.F. Harbage, Inheritance of freezing resistance in tuber bearing *Solanum* species: evidence for independent genetic control of nonacclimated freezing tolerance and cold acclimation capacity, Proc. Natl. Acad. Sci. U.S.A. 90 (1993) 7869–7873.

[4] M. Byrne, J.C. Murrell, J.V. Owen, E.R. Williams, G.F. Moran, Mapping of quantitative trait loci influencing frost tolerance in *Eucalyptus nitens*, Theor. Appl. Genet. 95 (1997) 975–979.

[5] R. Arora, L.J. Rowland, J.S. Lehman, C.C. Lim, G.R. Panta, N. Vorsa, Genetic analysis of freezing tolerance in blueberry (*Vaccinium* section *Cyanococcus*), Theor. Appl. Genet. 100 (2000) 690–696.

[6] G.T. Howe, P. Saruul, J. Davis, T.H.H. Chen, Quantitative genetics of bud phenology, frost damage, and winter survival in an $F_2$ family of hybrid poplars, Theor. Appl. Genet. 101 (2000) 632–642.

[7] K.D. Jermstad, D.L. Bassoni, N.C. Wheeler, T.S. Anekonda, S.N. Aitken, W.T. Adams, D.B. Neale, Mapping of quantitative trait loci controlling adaptive traits in coastal Douglas–fir. II. Spring and fall cold-hardiness, Theor. Appl. Genet. 102 (2001) 1152–1158.

[8] C.L. Guy, Cold acclimation and freezing stress tolerance: role of protein metabolism, Annu. Rev. Plant Physiol. Plant Mol. Biol. 41 (1990) 187–223.

[9] K. Shinozaki, K. Yamaguchi-Shinozaki, Molecular responses to drought and cold stress, Curr. Opin. Biotechnol. 7 (1996) 161–167.

[10] M.F. Thomashow, Plant cold acclimation: freezing tolerance genes and regulatory mechanisms, Annu. Rev. Plant Physiol. Plant Mol. Biol. 50 (1999) 573–599.

[11] M. Seki, M. Narusaka, H. Abe, M. Kasuga, K. Yamaguchi-Shinozaki, P. Carninci, Y. Hayashizaki, K. Shinozaki, Monitoring the expression pattern of 1300 *Arabidopsis* genes under drought and cold stresses by using a full-length cDNA microarray, Plant Cell 13 (2001) 61–72.

[12] S. Fowler, M.F. Thomashow, *Arabidopsis* transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway, Plant Cell 14 (2002) 1675–1690.

[13] A.L. Dhanaraj, J.P. Slovin, L.J. Rowland, Analysis of gene expression associated with cold acclimation in blueberry floral buds using expressed sequence tags, Plant Sci. 166 (2004) 863–872.

[14] A.L. Dhanaraj, N.W. Alkharouf, H.S. Beard, I.B. Chouikha, B.F. Matthews, H. Wei, R. Arora, L.J. Rowland, Major differences observed in transcript profiles of blueberry during cold acclimation under field and cold room conditions, Planta 225 (2007) 735–751.

[15] K. Singh, R.C. Foley, L. Onate-Sanchez, Transcription factors in plant defense and stress responses, Curr. Opin. Plant Biol. 5 (2002) 430–436.

[16] M.M. Muthalif, L.J. Rowland, Identification of dehydrin-like proteins responsive to chilling in floral buds of blueberry (*Vaccinium*, section *Cyanococcus*), Plant Physiol. 104 (1994) 1439–1447.

[17] T.A. Wilkins, L.B. Smart, Isolation of RNA in plant tissues, in: P.A. Krieg (Ed.), A Laboratory Guide to RNA Isolation, Analysis and Synthesis, Wiley-Liss, Inc., 1996, pp. 21–41.

[18] S.F. Altschul, W. Gish, W. Miller, E.W. Myers, D. Lipman, Basic local alignment search tool, J. Mol. Biol. 215 (1990) 403–410.

[19] M. Riley, Functions of gene products in *E. coli*, Microbiol. Rev. 57 (1993) 862–952.

[20] H.W. Mewes, K. Albermann, M. Bahr, D. Frishman, A. Gleissner, J. Hani, K. Heumann, K. Klein, A. Maierl, S.G. Oliver, et al., Overview of the yeast genome, Nature 387 (Suppl.) (1997) 7–65.

[21] M. Bevan, I. Bancroft, E. Bent, K. Love, P. Pittanelli, H. Goodman, C. Dean, R. Bergkamp, W. Dirkse, M. Van Staveran, et al., Analysis of 1.9 Mb of contiguous sequence from chromosome 4 of *Arabidopsis thaliana*, Nature 391 (1998) 485–488.
[22] E. Ablett, G. Seaton, K. Scott, D. Shelton, M.W. Graham, P. Baverstock, L. Slade Lee, R. Henry, Analysis of grape ESTs: global gene expression patterns in leaf and berry, Plant Sci. 159 (2000) 87–95.
[23] M.F. Vieweg, N. Hohnjec, H. Kuster, Two genes encoding different truncated hemoglobins are regulated during root nodule and arbuscular mycorrhiza symbioses of *Medicago truncatula*, Planta 220 (2005) 757–766.
[24] I. Adamska, I. Ohad, K. Kloppstech, Synthesis of the early light-inducible protein is controlled by blue light and related to light stress, Proc. Natl. Acad. Sci. U.S.A. 89 (1992) 2610–2613.
[25] M. Heddad, I. Adamska, Light stress-regulated two-helix proteins in *Arabidopsis thaliana* related to the chlorophyll *a/b*-binding gene family, Proc. Natl. Acad. Sci. U.S.A. 97 (2000) 3741–3746.
[26] G. Meyer, K. Kloppstech, A rapidly light-induced chloroplast protein with a high turnover coded for by pea nuclear DNA, Eur. J. Biochem. 138 (1984) 201–207.
[27] I. Adamska, ELIPs: light induced stress proteins, Physiol. Plant 100 (1997) 794–805.
[28] C. Hutin, L. Nussaume, N. Moise, I. Moya, K. Kloppstech, M. Havaux, Early light-induced proteins protect *Arabidopsis* from photooxidative stress, Proc. Natl. Acad. Sci. U.S.A. 100 (2003) 4921–4926.
[29] M.A. Hannah, A.G. Heyer, D.K. Hincha, A global survey of gene regulation during cold acclimation in *Arabidopsis thaliana*, PLoS Genet. 1 (2005) 179–196.
[30] I.V. Hall, F.R. Forsyth, R.J. Newberry, Effect of temperature on flower bud and leaf anthocyanin formation in the lowbush blueberry, Hortscience 5 (1970) 272–273.
[31] E.J. Stockinger, S.J. Gilmour, M.F. Thomashow, *Arabidopsis thaliana* CBF1 encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a *cis*-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit, Proc. Natl. Acad. Sci. U.S.A. 94 (1997) 1035–1040.
[32] S.J. Gilmour, D.G. Zarka, E.J. Stockinger, M.P. Salazar, J.M. Houghton, M.F. Thomashow, Low temperature regulation of the *Arabidopsis* CBF family of AP2 transcriptional activators as an early step in cold-induced *COR* gene expression, Plant J. 16 (1998) 433–442.
[33] Q. Liu, M. Kasuga, Y. Sakuma, H. Abe, S. Miura, K. Yamaguchi-Shinozaki, K. Shinozaki, Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in *Arabidopsis*, Plant Cell 10 (1998) 1391–1406.
[34] Z.K. Shinwari, K. Nakashima, S. Miura, M. Kasuga, M. Seki, K. Yamaguchi-Shinozaki, K. Shinozaki, An *Arabidopsis* gene family encoding DRE/CRT binding proteins involved in low-temperature-responsive gene expression, Biochem. Biophys. Res. Commun. 250 (1998) 161–170.
[35] C.J. Weiser, Cold resistance and injury in woody plants, Science 169 (1970) 1269–1278.
[36] C.M.F. Wake, A. Fennell, Morphological, physiological and dormancy responses of three Vitis genotypes to short photoperiod, Physiol. Plant 109 (2000) 203–210.
[37] A. Kasprzewska, Plant chitinases—regulation and function, Cell. Mol. Biol. Lett. 8 (2003) 809–824.
[38] M.A. Naranjo, J. Forment, M. Roldan, R. Serrano, O. Vicente, Overexpression of *Arabidopsis thaliana LTL1*, a salt-induced gene encoding a GDSL-motif lipase, increases salt tolerance in yeast and transgenic plants, Plant Cell Environ. 29 (2006) 1890–1900.
[39] J. Borecky, F.T. Nogueira, K.A. de Oliveira, I.G. Maia, A.E. Vercesi, P. Arruda, The plant energy-dissipating mitochondrial systems: depicting the genomic structure and the expression profiles of the gene families of uncoupling protein and alternative oxidase in monocots and dicots, J. Exp. Bot. 57 (2006) 849–864.
[40] N.A. Delk, K.A. Johnson, N.I. Chowdhury, J. Braam, CML24, regulated in expression by diverse stimuli, encodes a potential $Ca^{2+}$ sensor that functions in responses to abscisic acid, daylength, and ion stress, Plant Physiol. 139 (2005) 240–253.
[41] A. Mukhopadhyay, S. Vij, A.K. Tyagi, Overexpression of a zinc-finger protein gene from rice confers tolerance to cold, dehydration, and salt stress in transgenic tobacco, Proc. Natl. Acad. Sci. U.S.A. 101 (2004) 6309–6314.
[42] S. Davletova, K. Schlauch, J. Coutu, R. Mittler, The zinc-finger protein Zat12 plays a central role in reactive oxygen and abiotic stress signaling in *Arabidopsis*, Plant Physiol. 139 (2005) 847–856.
[43] J.Q. Wen, K. Oono, R. Imai, Two novel mitogen-activated protein signaling components, OsMEK1 and OsMAP1, are involved in a moderate low-temperature signaling pathway in rice, Plant Physiol. 129 (2002) 1880–1891.
[44] E.W. Hwang, K.A. Kim, S.C. Park, M.J. Jeong, M.O. Byun, H.B. Kwon, Expression profiles of hot pepper (*Capsicum annum*) genes under cold stress conditions, J. Biosci. 30 (2005) 657–667.
[45] T. Urao, K. Yamaguchi-Shinozaki, S. Urao, K. Shinozaki, An *Arabidopsis* myb homolog is induced by dehydration stress and its gene product binds to the conserved MYB recognition sequence, Plant Cell 5 (1993) 1529–1539.
[46] N.A. Ramsay, A.R. Walker, M. Mooney, J.C. Gray, Two basic-helix-loop-helix genes (MYC-146 and GL3) from *Arabidopsis* can activate anthocyanin biosynthesis in a white-flowered *Matthiola incana* mutant, Plant Mol. Biol. 52 (2003) 679–688.
[47] Y. Hyun, I. Lee, KIDARI, encoding a non-DNA binding bHLH protein, represses light signal transduction in *Arabidopsis thaliana*, Plant Mol. Biol. 61 (2006) 283–296.
[48] H. Abe, T. Urao, T. Ito, M. Seki, K. Shinozaki, K. Yamaguchi-Shinozaki, *Arabidopsis* AtMYC2 (bHLH) and AtMYB2 (MYB) function as transcriptional activators in abscisic acid signaling, Plant Cell 15 (2003) 63–78.
[49] V. Chinnusamy, M. Ohta, S. Kanrar, B.H. Lee, X. Hong, M. Agarwal, J.K. Zhu, ICE1: a regulator of cold-induced transcriptome and freezing tolerance in *Arabidopsis*, Genes Dev. 17 (2003) 1043–1054.
[50] G. Serino, T. Tsuge, S. Kwok, M. Matsui, N. Wei, X.W. Deng, *Arabidopsis* cop8 and fus4 mutations define the same gene that encodes subunit 4 of the COP9 signalosome, Plant Cell 10 (1999) 1967–1980.
[51] A. Cantero, S. Barthakur, T.J. Bushart, S. Chou, R.O. Morgan, M.P. Fernandez, G.B. Clark, S.J. Roux, Expression profiling of the *Arabidopsis* annexin gene family during germination, de-etiolation and abiotic stress, Plant Physiol. Biochem. 44 (2006) 13–24.
[52] C.D. Buchanan, S. Lim, R.A. Salzman, I. Kagiampakis, D.T. Morishige, B.D. Weers, R.R. Klein, L.H. Pratt, M.M. Cordonnier-Pratt, P.E. Klein, J.E. Mullet, Sorghum bicolor's transcriptome response to dehydration, high salinity and ABA, Plant Mol. Biol. 58 (2005) 699–720.
[53] J.H. Ko, S.H. Yang, K.H. Han, Upregulation of an *Arabidopsis* RING-H2 gene, XERICO, confers drought tolerance through increased abscisic acid biosynthesis, Plant J. 47 (2006) 343–355.